Report for 2002MN1B: Characterization of Nitrifying Bacterial Populations in Wastewater Treatment Bioreactors

There are no reported publications resulting from this project.

Report Follows:

Characterization of Nitrifying Bacterial Populations in Wastewater Treatment Bioreactors

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Summary

Excessive nitrogen loading to the Mississippi River basin has been recently linked to the development of a large hypoxic zone in the northern Gulf of Mexico. As a result, there is renewed interest in achieving complete nitrogen removal from municipal and industrial wastewater. One of the most critical challenges that must be addressed before complete nitrogen removal can be consistently achieved is to reduce and eliminate upsets in the nitrification process. Nitrifying bacteria are well known to be susceptible to numerous factors such as temperature, pH, and toxic compounds. One of the problems with eliminating nitrification upsets is that there is a virtually complete lack of knowledge regarding the community dynamics of nitrifying bacteria. The research proposed herein would track both nitrifier community structure and total nitrifier biomass at a municipal wastewater treatment facility for a period of one year. To date, we have collected samples from the aeration tanks of the Metropolitan Wastewater Treatment Facility twice per month from June 2002 through February 2003. We plan to continue collecting samples until May 2003. From these samples we will measure total microbial biomass (as particulate protein), total nitrifying bacterial biomass (as *amoA* gene copy number), and identify the physiologically relevant nitrifying bacteria using a nested PCR-DGGE approach. These results will be compared to influent and effluent biochemical oxygen demand concentrations, temperature, pH, dissolved oxygen concentrations, and ammonia concentrations at the treatment facility at the times of sample collection. This research will identify the specific nitrifying bacteria that are associated with excellent nitrification efficiency, thereby leading towards the development of specific operational strategies to promote the growth of these nitrifying bacteria. In conclusion, this research will be an important step in reducing the total nitrogen loading to the Mississippi River basin and the northern Gulf of Mexico.

Introduction

Nitrogenous pollutant removal from wastewater has received renewed interested recently due to a large hypoxic zone that has developed over the last several decades in the northern Gulf of Mexico. This large area (> 8,000 km^2) has exhibited severely depleted dissolved oxygen concentrations (DO < 2 mg L^{-1}) near the Mississippi River delta since the 1950s and 1960s (Rabalais *et al*., 2001). Nitrogenous compounds, particularly nitrate, have long been suspected to be the cause of this hypoxic zone that has an adverse affect on aquatic life and commercial fisheries. River basins in southern Minnesota, Iowa, Illinois, Indiana, and Ohio have been identified as the primary N-sources (Goolsby *et al*., 2001). Preliminary efforts are currently underway to reduce nutrient loading to the Mississippi River basin, including the construction of wetlands, riparian forests, and flood plains (Boesch and Brinsfield, 2000). These efforts will undoubtedly be extended to the regulation of municipal and industrial wastewater discharges.

Numerous process designs exist to adequately remove nitrogenous pollutants from wastewater. Operational control of these processes is hindered by inconsistent nitrification performance. Nitrifying bacteria are sensitive to pH, temperature, dissolved oxygen, and toxic compounds. Nitrifying bacteria are also slow-growing, so their recovery from perturbation is slow. One problem with controlling nitrification during wastewater treatment is an inadequate knowledge of the nitrifying bacterial population dynamics. Numerous cultivation-based techniques have been developed to quantify the biomass densities of nitrifying and denitrifying communities (e.g., the most-probable-number assay) (APHA, 1992), however these methods are infamous for underestimating the actual population density by an order of magnitude or more (Amann *et al*., 1995). Furthermore, cultivation-based assays are time-consuming because nitrifying bacteria grow slowly and fail to provide relevant data on microbial community structure.

The goal of this research is to examine the nitrifying bacterial community at a full-scale municipal wastewater treatment plant over a period of one year. This objective will be achieved through the application of novel molecular-genetic techniques for the analysis of bacterial communities without cultivation. The total number of nitrifying bacteria will be determined using a quantitative real-time polymerase chain reaction (PCR). The types of nitrifying bacteria will be determined using denaturing gradient gel electrophoresis.

Methods

General Approach

The first phase of the proposed research is to optimize the quantitative competitive PCR approach for nitrifying bacteria. Concomitant to this laboratory work, samples are collected from the wastewater treatment facility will be used to characterize both the amount and type of nitrifying bacteria present in the wastewater treatment bioreactor. These samples are stored at –20°C until the DNA is extracted and then subjected to either quantitative PCR or denaturing gradient gel electrophoresis.

Sample Collection, DNA Extraction and Quantification

Bioreactor samples (10 mL) are collected in triplicate from the Metropolitan Wastewater Treatment Plant (St. Paul, MN), placed on ice, and immediately returned to the University of Minnesota. Samples are then centrifuged, the supernatant is removed, and the cell pellet is resuspended in 1 mL of lysis buffer (120 mM sodium phosphate buffer, pH 8.0, 5% sodium dodecyl sulfate). Samples in lysis buffer are then stored at –20°C until processed further. Samples will be collected once every two weeks from June 2002 through May 2003.

Total genomic DNA is purified from samples using the FastDNA Spin Kit per manufacturer's instructions (Qbiogene). Samples undergo three freeze-thaw cycles followed by bead-beating to lyse cells. Extracted DNA is quantified by staining with Hoescht dye 33258 and measuring fluorescence output on a TD-700 fluorometer (Turner Designs; Sunnyvale, CA); results are correlated to a standard curve of calf thymus DNA.

Total Biomass Measurement

Once the population densities of nitrifying bacterial populations have been quantified, it will be important to correlate these values to total biomass levels. Cellular protein is extracted (Herbert *et al*., 1971) and measured according to the Lowry method (Lowry *et al*., 1951) using bovine serum albumin (BSA) as a protein standard.

Quantification of Nitrifying Bacteria

Two different approaches may be undertaken to quantify the number of ammonia-oxidizing bacteria in wastewater treatment samples. The first method is quantitative competitive PCR (cPCR), which works by including a known amount of artificially synthesized DNA (i.e., competitor) in a PCR reaction; the concentration of an unknown sample is determined when the amplification of the unknown and competitor are equal. We will apply the cPCR technique described by Dionisi *et al*. (2002) to quantify *amoA* gene copy number. Quantitative real-time PCR will also be attempted. This method works by measuring the amount of PCR product generated over time during the thermal cycle program and comparing results to known standards. The advantage of real-time PCR compared to cPCR is that less labor intensive and a large number of samples can be processed simultaneously (instead of individually as with cPCR).

Nitrifying Community Structure

Bacterial community shifts will be detected by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments specific for nitrifying bacteria. PCR-DGGE is a relatively simple method to detect qualitative shifts in bacterial community structure (Muyzer *et al.*, 1993). A detailed description of the PCR-DGGE approach that will be used has been presented elsewhere (LaPara *et al.*, 2000). The 16S rRNA gene of the known nitrifying bacteria will be amplified by PCR using the CTO189f and CTO654r primers (Kowalchuk *et al.*, 1993). A second PCR reaction will then be applied using primers PRBA338F and PRUN518R with a GC-clamp attached to the forward primer.

DGGE will be performed on a D-Code apparatus (BioRad; Hercules, CA) using a denaturing gradient ranging from 30 to 55% denaturant (100% denaturant contains 7 M urea and 40% formamide). Following electrophoresis, the gel will be stained with SYBR Green I (Molecular Probes; Eugene, OR), then visualized on a UV transilluminator and photographed with a digital CCD camera (BioChemi System; UVP, Inc; Upland, CA). Promiscuous bands will be excised, purified by repeated rounds of PCR-DGGE, and sequenced at the Advanced Genetic Analysis Center at the University of Minnesota.

Results to date

Sample Collection

Sample collection began in June 2002 and has continued to present (March 2003). Samples have been properly preserved and analysis will begin once the entire set of samples has been collected (anticipated: May 2003).

Total Biomass Measurement

Total biomass has been quantified from samples collected to date. Total biomass concentrations ranged from 400 mg/L to 1100 mg/L (as protein) (data not shown).

Quantification of Nitrifying Bacteria

Using samples from other projects, a competitive quantitative PCR technique has been optimized for quantification of *amoA* gene copy number (Figure 1). Band intensities shown in Figure 1 were quantified and compared to estimate the concentration at which target (Band A) and competitor (Band B) were equal (i.e., the number of *amoA* genes present in the sample).

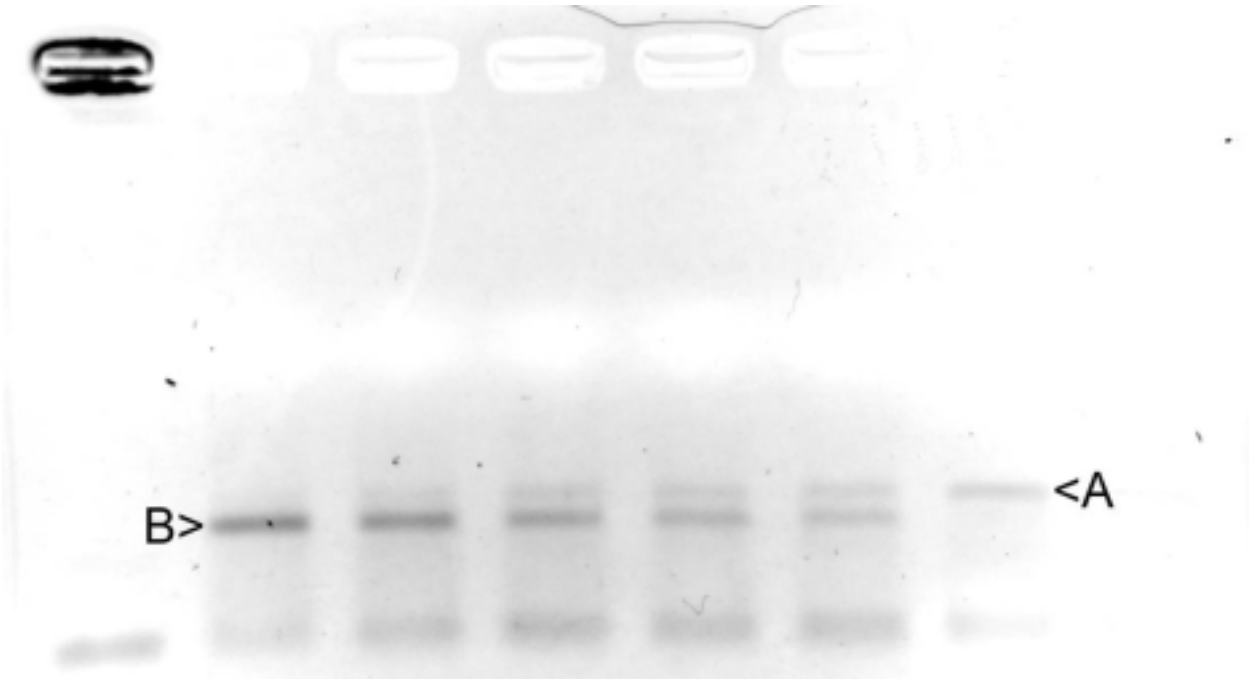

Figure 1: Competitive PCR of *amoA* genes.

Nitrifying Community Structure

Using samples from other projects, a nested PCR-DGGE technique has been developed for characterization of nitrifying bacterial communities (Figure 2). Of the five bands identified in Figure 2, DNA sequence analysis showed that three of these bands were phylogenetically related to known nitrifying bacteria. Two were *Nitrosomonas*-like (Bands A and B) and one was *Nitrosospira*-like (Band E). Other bands were experimental artifacts not likely to be associated with nitrifying bacterial populations.

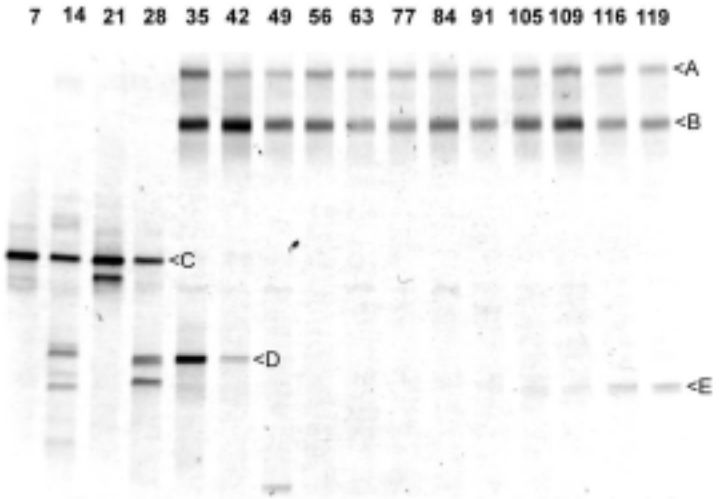

Figure 2: Nested PCR-DGGE for the analysis of nitrifying bacteria.

Summary of findings

The project is still on-going and sample collection is still underway. Methods to quantify nitrifying bacteria by competitive polymerase chain reaction have been successfully developed. A nested PCR-DGGE technique has also been developed to track nitrifying bacterial community structure. The principal investigator anticipates that substantive results will be rapidly generated once the sample collection period is complete.

References

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Publications associated with the project

None to date.

Students supported by the project -- graduate (MS, PhD) and undergradute

Sudeshna Ghosh (January 1, 2003 – date)

Department of Civil Engineering, University of Minnesota
Degree being sought: Masters of Science

Shelby Stanek
Department of Civil Engineering, University of Minnesota
Undergraduate

Awards and achievements resulting from your project
None to date.

Seminar or poster presentations resulting from your project
None to date.